Research article

Sub-inhibitory concentrations of vancomycin prevent quinolone-resistance in a penicillin-resistant isolate of Streptococcus pneumoniae

Philippe Cottagnoud*[1], Jose M Entenza[2], Marianne Cottagnoud[3], Yok-Ai Que[2], Philippe Moreillon[2] and Martin G Täuber[4]

Address: [1]Department of Internal Medicine, Inselspital, Freiburgstrasse, CH-3010 Berne, Switzerland, [2]Division of Infectious Diseases, Department of Internal Medicine, Centre Hospitalier Universitaire Vaudois, CH-1011 Lausanne, Switzerland, [3]Department of Internal Medicine, Spital Bern Ziegler, Morillonstrasse 75-91, CH-3007 Berne, Switzerland and [4]Institute for Infectious Diseases, University of Berne, CH-3010 Berne, Switzerland

E-mail: Philippe Cottagnoud* - pcottagn@insel.ch; Jose M Entenza - jose.entenza@chuv.hospvd.ch; Marianne Cottagnoud - marianne.cottagnoud@ziegler.ch; Yok-Ai Que - yok-ai.que@chuv.hopsvd.ch; Philippe Moreillon - philippe.moreillon@chuv.hospvd.ch; Martin G Täuber - martin.taeuber@ifik.unibe.ch

*Corresponding author

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Abstract

Background: The continuous spread of penicillin-resistant pneumococci represents a permanent threat in the treatment of pneumococcal infections, especially when strains show additional resistance to quinolones. The main objective of this study was to determine a treatment modality impeding the emergence of quinolone resistance.

Results: Exposure of a penicillin-resistant pneumococcus to increasing concentrations of trovafloxacin or ciprofloxacin selected for mutants resistant to these drugs. In the presence of sub-inhibitory concentrations of vancomycin, development of trovafloxacin-resistance and high-level ciprofloxacin-resistance were prevented.

Conclusions: Considering the risk of quinolone-resistance in pneumococci, the observation might be of clinical importance.

Background

Since the late seventies, the worldwide emergence of penicillin-resistant pneumococci has jeopardized the efficacy of β-lactam antibiotics, in life threatening infections such as meningitis or pneumonia [1]. Moreover, penicillin-resistant pneumococci are often resistant to multiple other drugs, thus restricting the choice of alternative compounds [2]. Therefore, new anti-pneumococcal drugs should combine the abilities to (i) rapidly inhibit and kill the target organisms, (ii) penetrate in various body compartments, including the cerebrospinal fluid, and (iii) impede resistance development against the new compounds. Newer quinolones with good anti gram-positive activity, including trovafloxacin, might fulfill these criteria. However, quinolone-resistant pneumococci can arise by acquisition of only one or two mutations in the genes of the quinolone targets, i.e., the topoisomerase IV (*parC* and *parE*) and the gyrase (*gyrA* and *gyrE*) [3,4,5,]. This mechanism of resistance is much less complicated than acquisition of resistance to penicillin by transformation with major gene sequences for PBPs. One would therefore expect that the activity of

quinolones against pneumococci is already jeopardized. Indeed, recent data support this notion [3].

Recently, we observed that addition of vancomycin to trovafloxacin improved the bactericidal activity of the quinolone against penicillin-resistant pneumococci both *in vitro* and in rabbits with experimental meningitis [6]. We now demonstrate that sub-inhibitory concentrations of vancomycin ($^1/_4$ MIC: 0.03 mg/L), that did not affect the quinolone MIC per se, also drastically prevented resistance to ciprofloxacin, and totally prevented resistance to trovafloxacin. The observation deserves attention because it might be of clinical relevance.

Results

Repeated exposure of WB4 to stepwise increasing concentrations of either trovafloxacin or ciprofloxacin resulted in resistance development against both drugs. Figure 1 indicates that the MIC of trovafloxacin had increased by 32-fold (MIC 4 mg/L) after only five passages. Likewise, the MIC of ciprofloxacin increased 16-fold (8 mg/L) after only three antibiotic passages. In sharp contrast, addition of sub-inhibitory concentrations ($^1/_4$ the MIC: 0.03 mg/L) of vancomycin to trovafloxacin completely prevented the emergence of mutants resistant to this drug, and the MIC of trovafloxacin remained unchanged for up to eight cycles (Figure 1). Moreover, addition of vancomycin to ciprofloxacin also reduced resistance development against this compound, albeit not to the same extent as for trovafloxacin. Indeed, a slight increase to 2-fold the MIC (1 mg/L) was observed in this experiment (Figure 2). Addition of $^1/_4$ the MIC of vancomycin did not affect the MIC of the test quinolones and resistance to vancomycin has not been observed in quinolone-resistant mutants either (Table 1).

As previously described, there was a certain amount of cross-resistance between the two test quinolones. Table 1 indicates that resistance to trovafloxacin was accompanied by a parallel increase in the ciprofloxacin MIC (from 0.5 mg/L to > 32 mg/L). On the other hand, selection of resistance with ciprofloxacin only marginally affected the MIC of trovafloxacin (from 0.12 to 0.25 mg/L).

The difference between these cross-resistance patterns most likely relied in the specific mutations selected by the two drugs. Table 2 presents the mutations in the topoisomerase IV (*parC* and *parE*) and gyrase (*gyrA* and *gyrB*) genes observed in resistant mutants selected with either of the compounds. Trovafloxacin selected mutations in the *parC* and the *gyrA* genes. The *parC* mutation (Ser79→Phe) was previously described [11,12,13]. Two other *parE* (Asp435→Asn, and Ile460→Val) were recently observed in a clinical isolate of trovafloxacin-resistant pneumococcus [14], but did not appear in the present experiments. The *gyrA* mutation (Ser81→Phe)

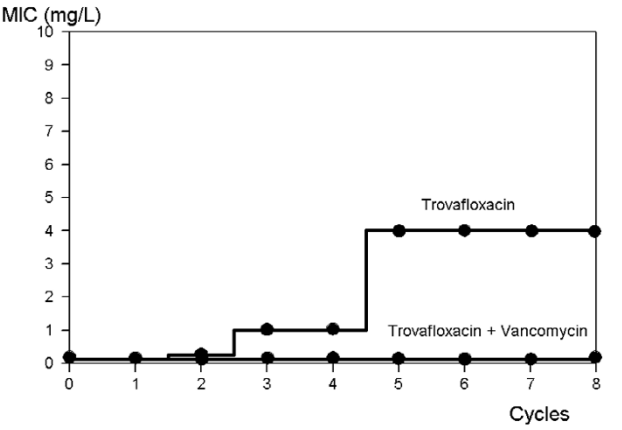

Figure 1
shows selection of trovafloxacin resistant mutants of *Streptococcus pneumoniae* WB4 exposed to stepwise increasing concentrations of trovafloxacin alone or in combination with sub-MIC concentration ($^1/_4$ MIC) of vancomycin.

has been reported as well [5]. This mutation resembles a

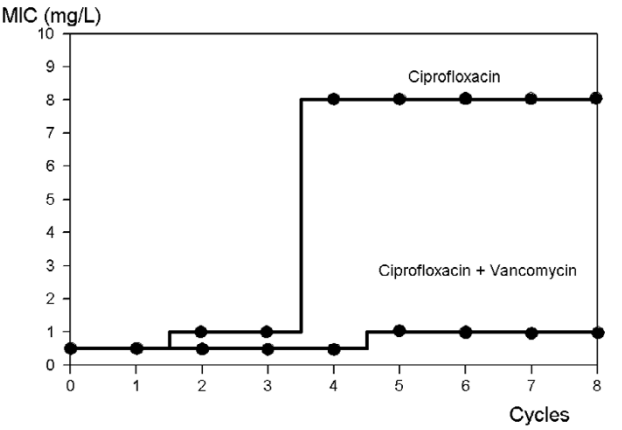

Figure 2
shows selection of ciprofloxacin resistant mutants of *Streptococcus pneumoniae* WB4 exposed to stepwise increasing concentrations of ciprofloxacin alone or in combination with sub-MIC concentration ($^1/_4$ MIC) of vancomycin.

gyrA (Ser83→Phe) mutation described in ciprofloxacin-resistant pneumococci [15], and is likely to be responsible for the cross-resistance pattern between trovafloxacin and ciprofloxacin.

In contrast, resistance to ciprofloxacin was somewhat different. The *parC* mutation (Ser79→Tyr) was relatively conserved when compared to the *parC* mutation selected

by trovafloxacin (Ser79→Phe). Indeed, both substitutions (Tyr and Phe) involve aromatic acids that differ only by one hydroxyl group. On the other hand, the *GyrB* mutation (Asp435→Glu) has been described in ciprofloxacin-resistant derivatives, but not in trovafloxacin-resistant clones [15]. Therefore, it is likely that this mutation cannot confer cross-resistance to trovafloxacin.

Discussion

Sub-inhibitory concentration of vancomycin prevented the selection of all these mutations, except for the low level resistance mutation to ciprofloxacin (Table 2). Since these vancomycin concentrations did not affect the quinolones' MICs, it was unlikely that mutation prevention was merely due to a combined bacteriostatic effect of the two drugs. An other conceivable explanation for this phenomenon might be an increased intracellular penetration of the quinolones by addition of the cell wall active antibiotic. This would lead to intracellular antibiotic levels above the mutant prevention concentration (MPC) impeding the emergence of mutations [16]. However, this hypothesis is less probable because one would expect a change of the MIC in presence of vancomycin (see Table 1). On the other hand, we did previously show that the combination of vancomycin with quinolones synergically increased the bactericidal effect of these drugs [6]. Therefore, resistance prevention might be due to improved bactericidal killing at the MIC and supra-MIC concentrations, thus lowering the bacterial population below the critical level that allows selection for chromosomal mutations (i.e., below 10^6-10^8 CFU). This was indeed the case both *in vitro* and in rabbits with experimental meningitis [6].

Table 1: MICs of trovafloxacin and ciprofloxacin alone and in combination with subinhibitory concentrations of vancomycin

	MIC(mg/L)				
	WB4	**WB4C**	**WB4T**	**WB4C+V**	**WB4T+V**
Ciprofloxacin	0.5	8	>32	1	1
Ciprofloxacin + Vancomycin (1/4 MIC)	0.5	8	>32	1	1
Trovafloxacin	0.12	0.25	4	0.25	0.12
Trovafloxacin + Vancomycin (1/4MIC)	0.12	0.25	4	0.25	0.12
Vancomycin	0.12	0.12	0.12	0.12	0.12

WB4: quinolone-susceptible but penicillin-resistant parent pneumococcus; **WB4 C:** ciprofloxacin-resistant derivative selected by passages on this drug; **WB4 T:** trovafloxacin-resistant derivative selected by passages on this drug; **WB4 C+V** or **WB4 T+V:** same as above cycled in presence of subinhibitory concentrations of vancomycin.

Table 2: Mutations in topoisomerase IV (*ParC* and *ParE*) and gyrase (*GyrA* and *GyrB*) genes before and after cyclic exposure to ciprofloxacin, trovafloxacin, or either of these drugs plus vancomycin in a penicillin-resistant pneumococcal strain

Strain	ParC	ParE	GyrA	GyrB
WB4	None	none	none	none
WB4C	Ser79 → Tyr	none	none	Asp435 → Glu
WB4T	Ser79 → Phe	none	Ser81 → Phe	none
WB4 C+V	Ser79 → Tyr	none	none	none
WB4 T+V	None	none	none	none

WB4: quinolone-susceptible but penicillin-resistant parent pneumococcus; **WB4 C:** ciprofloxacin-resistant derivative selected by passages on this drug; **WB4 T:** trovafloxacin-resistant derivative selected by passages on this drug; **WB4 C+V** or **WB4 T+V:** same as above cycled in presence of subinhibitory concentrations of vancomycin.

Conclusions

The data observed here are reminiscent of the synergic activity of cell wall active antibiotics and aminoglycosides in enterococci and other gram-positive pathogens. Although the mechanism of this synergism is not entirely clear, it is important both to prevent resistance and improve therapeutic efficacy in severe infections. A similar model could hold true with the combination of cell wall inhibitors and trovafloxacin or other quinolones in pneumococcal infections. Therefore, the present observation with vancomycin and quinolones might be of clinical relevance both for resistance prevention and treatment efficacy. Moreover, it opens the avenue to other drug combinations.

Materials and Methods

Antibiotics and MIC determination

Trovafloxacin was provided by Pfizer Inc. (Groton, Conn.), ciprofloxacin was purchased from Bayer AG (Wuppertal, Germany), and vancomycin was purchased from Eli Lilly (Geneva, Switzerland). WB4 is a penicillin-resistant isolate (MIC: 4 mg/L) serotype 6 originally isolated from a patient with pneumonia at the University Hospital of Berne, Switzerland, and was grown in C+Y medium [7]. MICs were determined by broth macrodilution methods [8]. The MIC was defined as the lowest concentration that inhibited visible growth after 12 and 24 h of incubation at 37°C.

Selection of quinolone-resistant derivatives in vitro

Experiments were designed to test the tendency of trovafloxacin and ciprofloxacin to select resistant strains in liquid cultures. Large inocula (10^7-10^8 CFU/ml) of WB4 were exposed to stepwise increasing concentrations of antibiotics [9]. Series of tubes containing twofold increasing concentrations of either trovafloxacin or ciprofloxacin were inoculated with WB4 (10^7-10^8 CFU/ml), as for the MIC determination. After 12 hours of incubation 0.1 ml samples from the tubes containing the highest antibiotic concentration and still showing turbidity were used to inoculate a new series of tubes containing antibiotic serial dilutions. The experiment was performed during eight cycles. The MIC was determined after each cycle.

In further series, the same experimental protocol was used but vancomycin was added in low concentrations (0.03 mg/L corresponding to $^1/_4$ MIC) to the tubes containing serial dilutions of either trovafloxacin or ciprofloxacin. After 12 hours of incubation MIC was determined as described above in tubes containing only either trovafloxacin or ciprofloxacin.

Preparation of chromosomal DNA, PCR amplification and DNA sequence analysis

Chromosomal pneumococcal DNA was prepared as described [10]. PCR-amplification of the *parC, parE, gyrA* and *gyrB* genes were performed according to a published method [11]. PCR-amplification was performed with a GeneAmp PCR System 9700 apparatus (Perkin Elmer). After amplification, PCR products were purified by using a QIAquick PCR purification kit (Quiagen AG, Basel, Switzerland). Nucleotide sequencing of the PCR amplicons was carried out by using the ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction kit according to the protocol of the manufacturer (Perkin Elmer). An ABI PRISM 377 DNA sequencer was used for sequencing. All testing was performed in duplicate.

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